Evaluation of the Antibacterial Properties of Lactobacillus acidophilus Metabolites against Oral Plaque Streptococci: An In vitro Study

Fatemeh Babadi¹,², Mansour Amin¹,³, Fatemeh Ahmadi Behbahani¹,²,⁴*

¹Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
²Department of Oral and Maxillofacial Medicine, School of Dentistry, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
³Department of Microbiology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
⁴Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

ABSTRACT
The bacterial plaque is one of the important factors in the destruction of teeth and periodontal tissues. Probiotics have been proved to be effective in the plaque reduction and gingival health maintenance. Lactic Acid Bacteria (LAB), especially lactobacilli are known for their antimicrobial activity. Most of the antimicrobial activity is due to the secondary metabolites produced by certain LAB strains. The aim of this study was to investigate the antibacterial effects of Lactobacillus acidophilus metabolites on common oral streptococci. In this in vitro investigation, the minimum inhibitory concentrations (MIC) of Lactobacillus acidophilus metabolites was assessed using a modified E test, against three plaque-forming bacteria, including Streptococcus mutans, Streptococcus sanguinis, and Streptococcus salivarius. The metabolites of Lactobacillus acidophilus were extracted using ethyl acetate. The L. acidophilus metabolites showed potent inhibitory activity against all tested strains. The MICs for S. mutans, S. sanguinis, and S. salivarius were 0.01875, 0.009 and 0.15 mg/ml, respectively. The findings of this study revealed that L. acidophilus produced compounds with a good antibacterial activity which may provide a basis for alternative therapies for the prevention and control of the oral plaque-forming bacteria.

Key words: Lactobacillus acidophilus, Probiotics, Oral plaque, Antibacterial properties

INTRODUCTION

The use of probiotics to increase and improve the human health is a long-standing proposal. In recent years, many studies have been conducted to confirm the anti-pathogenic effects of probiotics. Probiotics are defined as viable microorganisms that are beneficial to their host when administered in adequate amounts [1,2]. Some of the bacterial genera most used in probiotic preparations include Bifidobacterium, Lactobacillus, Saccharomyces and Leuconostoc [3]. The Lactobacillus genus contains various Gram-positive facultative anaerobic or microaerophilic rod-shaped microorganisms. They are a major part of the lactic acid bacteria (LAB) group.

Lactic acid bacteria (LAB) are among the probiotics that produce various antimicrobials components and bacteriocins. Due to their antimicrobial properties, probiotics have been suggested as prophylactic and therapeutic agents for oral diseases caused by bacteria found in dental plaque [3-7].

Dental plaque is a complex of microbial community found on the surface of teeth, embedded in a matrix of bacterial and salivary origin [8]. It is obvious that the specific nature of the microbiota of dental plaque is fundamental in the etiology and pathogenesis of dental caries and periodontitis [6,8]. Recently, Preventive approaches based upon the use of probiotic bacteria to reduce antibiotic use and develop novel treatments for oral diseases that do not involve conventional antimicrobial agents have been proposed [7,9].

Lactobacillus acidophilus, a member of the normal intestinal and vaginal flora of healthy humans, is one
of the most commonly suggested Lactobacillus species for use as a probiotic [10,11]. Some researchers from developed countries have reported significant in vitro inhibition of Gram negative and Gram-positive pathogenic bacteria by Lactobacillus acidophilus species, but similar studies and information about the inhibitory effects of Lactobacillus acidophilus against oral streptococci, which plays an important role in the development of dental plaques, from developing countries, such as Iran, are very limited [12,13].

Therefore, the aim of this study was to investigate the in vitro inhibitory effects of Lactobacillus acidophilus metabolites against the main plaque-forming streptococci including S. mutans, S. sanguinis, and S. salivarius.

**MATERIALS AND METHODS**

**Ethics**

The study was approved by the Research Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No: IR.AJUMS.REC.1397.079).

**Strains and culture conditions**

This study was conducted in the Microbiology Laboratory of the Department of Microbiology of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, during January to May 2018. All standard strains of Streptococcus mutans (PTCC 1683), S. sanguinis (PTCC 1449), S. salivarius (PTCC 1448) and L. acidophilus (PTCC 1643) were purchased from the Persian Type Culture Collection (PTCC) of the Iranian Research Organization for Science and Technology (IROST) in Tehran, Iran and cultured according to the manufacturer’s instructions. L. acidophilus was cultured in MRS (De Man, Rogosa, and Sharpe) broth (Merck, Darmstadt, Germany) for 48 hours under a 5% CO₂ atmosphere at 37°C. Streptococci strains were cultured on TSA (Triptic Soy Broth) (Merck, Darmstadt, Germany) with 5% defibrinated sheep blood and incubated for 24 h to 48 h at 37°C under aerobic conditions. The physiological features and purity of the isolates were analyzed by observation of their morphological characteristics using a light microscope, Gram staining, and a catalase test. Stock cultures of all strains were maintained at −20°C in 15% glycerol.

**Extraction of Lactobacillus acidophilus metabolites**

The colony of L. acidophilus, inoculated in MRS broth. After 3 days incubation, the MRS broth media containing bacteria was mixed with ethyl acetate (75:25) and agitated with a magnetic stirrer for 8 h. Then the media was allowed to settle for 30 min. Following the settlement, the solution was separated into two phases, which the supernatant was comprised of the extracted antimicrobial compound. The upper organic layer was separated using a separating funnel and centrifuged at 5000 rpm for 10 minutes. The ethyl acetate layer was then removed and transferred into a clean flask. The extract was pooled and dried in an incubator at 45°C. The yield from the extract was dissolved in methanol (Merck, Germany) and its pH was adjusted to 7 using NaOH for antimicrobial susceptibility testing [10,14].

**Antimicrobial susceptibility test (AST)**

Antibacterial activity on plates was done by the disc diffusion method (DDM). Initially, a bacterial suspensions equivalent to 0.5 McFarland standard inoculum were prepared and then inoculated onto Mueller-Hinton agar (MHA) (Merck, Germany) containing 5% sheep blood. Subsequently, 10 µl of the extract was loaded on to a sterilized 6 mm diameter Whatman no. 1 filter paper disc, and dried in sterile Petri dish at 37°C for 10 min. The dried discs were placed on the bacterial growth and diameter of inhibition zone (DIZ) around each disc was measured after 24 h incubation at 37°C. The antagonistic activity of the L. acidophilus was measured in triplicates and the data have been presented in Table 1.

The minimal inhibitory concentration (MIC) of L. acidophilus metabolite substance determined using an improved modified E test method (AB Biodisk, Solna, Sweden) in order to show the antimicrobial activity of L. acidophilus [10,14,15]. In the improved E test, several AB Biodiscs impregnated with different dilutions of the extracts were used instead of strips. In fact, it was a simulated version of the standard E test [15]. The S. mutans, S. sanguinis and S. salivarius suspensions of freshly grown cultures were adjusted to a density of 10⁴ cells/mL, corresponding to 68%-82% transmittance at 530 nm. The plate of Mueller-Hinton agar (MHA) with 5% sheep blood was inoculated by dipping a sterile cotton swab into the suspension and streaking it across the agar surface in three directions. The plates were dried at an ambient temperature for 15 min before applying the discs. Six sterile discs (6 mm) were impregnated with 10 µl of six different concentrations (0.15, 0.075, 0.0375, 0.01875, 0.009 and 0.004 mg/ml) of the serially diluted metabolite in methanol and placed in a line on the agar surface. The plates were incubated for 24 h at 37°C. The MIC values were read as the antimicrobial concentrations at the points where dense colonial growth intersected the discs [15]. The test was performed in triplicate for each strain.

**RESULTS**

The antimicrobial activity of the extracted metabolites from L. acidophilus (PTCC 1643) strain was assessed against three oral streptococci in Table 1. The DDM test showed the largest zone of inhibition (18 mm) against the Streptococcus sanguinis. The least zone of inhibition, 8 mm was shown against S. Salivarius. The findings of the DDM and modified E test showed a potent inhibitory activity of the L. acidophilus metabolites against all tested strains in Figure 1. The MICs of metabolites compound extracted from L. acidophilus were 0.01875, 0.009, and
Furthermore, Clostridium perfringens Shigella sonnei agents against pathogens such as Enterococcus faecium, Staphylococcus aureus, Staphylococcus epidermis, and Bacillus subtilis in the agar overlay method after 24 h incubation at 30°C [22]. In another study, Abdulla investigated the antimicrobial activity of L. acidophilus and revealed the highest inhibitory activity against B. subtilis, Pseudomonas aerogenosa, Streptococcus pyogenes, Proteus vulgaris, Staphylococcus aureas, and Aeromonas hydrophila [23]. Mahmood et al. reported that different strains of L. acidophilus cultured from a native milk product exerted inhibitory effects against food borne pathogens [24]. Jabbari et al. reported that the L. acidophilus isolated from traditional doogh samples which collected from the Tabriz region, Iran had a growth inhibitory activity against Staphylococcus aureus, Shigella dysenteriae, and Escherchia coli [25]. Because of the therapeutic potentiality of probiotic lactobacilli against MDR bacterial infection, they are one such microorganisms of choice to use as the remedial agents in combination with antibiotics [26]. As has been reported by Bassyouni et al. the L. acidophilus-ciprofloxacin combined therapy against infective gastroenteritis was effective in terms of decreasing the ciprofloxacin (CIP) dose and hence the CIP adverse effect on the balance of microbial flora in human intestine [27].

Table 1: Results of disc diffusion test of Lactobacillus acidophilus metabolite against indicator strains

<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>DIZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus mutans</td>
<td>12</td>
</tr>
<tr>
<td>Streptococcus sanguinis</td>
<td>18</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>8</td>
</tr>
</tbody>
</table>

Note: Values refer to the diameter of inhibition zone (in mm) and represent mean (average) of triplicates

0.15 mg/ml for S. mutans, S. sanguinis, and S. salivarius, respectively (Table 2). The metabolite extract exhibited the greatest effect on S. Sanguinis. Among the tested strains, S. salivarius showed the highest resistance to metabolites extract.

**DISCUSSION**

The present study aimed to determine whether the L. acidophilus (PTCC 1643) metabolites were able to exert an antagonistic effect against oral streptococci by disc diffusion and modified E test methods. In vitro experiment has revealed that selected L. acidophilus strain is effective against oral streptococci. Previous studies from different countries have been shown the important role of lactobacilli as potential antibacterial agents against pathogens such as Enterococcus faecium, Shigella sonnei and Clostridium perfringens [12,13,15].

Furthermore, Lactobacillus acidophilus is among the various lactobacilli that have been studied for their antibacterial effects in previous investigations [16]. Mohankumar et al. claimed that L. acidophilus had antagonist effects on the growth of Proteus spp. that is associated to the bacteriocin production [17]. The microbial inhibitory activity of lactobacilli can be probably attributed to the production of several antimicrobial compounds like bacteriocin, organic acid and hydrogen peroxide [18]. Dental caries and periodontal diseases are a major public health challenge in all countries of the world. The oral streptococci such as Streptococcus mutans play the major role in dental caries and periodontal diseases pathogenicity [6,7].

Because of the increasing antibiotic resistance of bacteria, new methods such as probiotic therapy for decreasing of oral cavity pathogens must be investigated. Lactobacillus acidophilus was investigated in this study because of its known probiotic potential [11]. According to our results, it is cleared that the (PTCC 1643) can inhibit the growth of streptococcal strains. Our study confirmed the results obtained by Khanafar et al. in whose study L. acidophilus (PTCC 1643) had the inhibitory effect on S. mutans isolated from samples of gingival dental plaque and caries lesions in adults [19]. Regarding the L. acidophilus antibacterial properties, research conducted by Tahmousp et al. showed that L. acidophilus was able to reduce the plaque formation of oral Streptococci [20]. In this study, the metabolite extract of L. acidophilus showed the best inhibitory effect on S. Sanguinis. Consistent with our findings, Miller et al. reported that the addition of the lactobacilli to cultures of Streptococcus sanguinis resulted in more inhibition of plaque formation when compared with pure cultures of Streptococcus sanguinis [21]. A survey by Aween et al. aimed at studying the antibacterial activity of L. acidophilus strains isolated from honey marketed in Malaysia, showed inhibitory activity against multiple antibiotic resistant’s Staphylococcus aureus, Staphylococcus epidermis, and Bacillus subtilis in the agar overlay method after 24 h incubation at 30°C [22].

Our study confirmed the results obtained by Khanafari et al. in whose study L. acidophilus (PTCC 1643) can inhibit the growth of S. Sanguinis. Among the tested strains, S. salivarius showed the highest resistance to metabolites extract.
Several studies have been conducted on the antibacterial potential of *lactobacilli*, which showed various results. There are some dissimilarities and contradictions between the results, indicating that the efficacy of *lactobacilli* products is significantly affected by many factors such as the method of metabolite extraction, antibacterial assay method and conditions, and the source of tested strain. In the present study, ethyl acetate was used to obtain the *Lactobacillus* extract. In other studies, researchers used the filtered supernatant fluids from MRS liquid medium containing *Lactobacillus* cells as antimicrobial extract [15]. In our study, Sodium hydroxide (NaOH) was added to the extract in order to neutralize its pH. Therefore, the antimicrobial activity of the extract was not related to its acidic nature.

However, the results obtained in this study can serve as a guide for further clinical investigations, in view of the limitations of in vitro studies, in vivo survey and further research using more complex techniques are needed to support the efficacy of *L. acidophilus* as an antibacterial probiotic against cariogenic pathogens.

**CONCLUSION**

This work highlights the antimicrobial potential of *L. acidophilus* metabolite in vitro. The results of the study allow us to suggest further research to introduce *L. acidophilus* for use in the prevention and control of dental plaque formation and other dental diseases.

**ACKNOWLEDGMENTS**

This research is a part of Doctor of Dentistry thesis of Fatemeh Ahmadi Behbahani, which was approved in Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Our appreciation goes to Vice Chancellor for Research affairs and the Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran for their financial (Grant No: OG-9708) and executive support.

**CONFLICT OF INTEREST**

The authors’ declares that they have no conflict of interest.

**REFERENCES**

19. Khanafari A, Hosseini Porgam S, Tajabadi Ebrahimi M. Investigation of probiotic chocolate...